PROTEIN SYNTHESIS AND INDUCTION OF THE HYPERSENSITIVE RESPONSE IN THE *Capsicum annuum*-*Xanthomonas vesicatoria* HOST-PARASITE SYSTEM

BY

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Abstract of Dissertation Presented to the Graduate Council
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Protein synthesis during compatible and incompatible interactions of Capsicum annuum L. (pepper) and Xanthomonas vesicatoria Doidge (Dows.) was determined. The relationship of the effects of chloramphenicol and streptomycin sulfate on protein synthesis to the length of the induction period of the bacterial-induced hypersensitive response (HR) was also investigated.

Two isolates of X. vesicatoria (XV), XV69-20 and XV70-3, were assumed to differ only in the gene(s) governing avirulence in specific near-isogenic C. annuum hosts. Host pepper line, 10-R, was compatible with isolate XV70-3, but was incompatible with XV69-20. Both isolates were compatible with the other pepper host, Early Calwonder (ECW). A half-leaf experimental design was used in which XV69-20 or XV70-3 was injected into one-half of a pepper leaf.

Protein synthesis was determined by measurement of the incorporation of a ^{14}C-amino acid mixture into trichloroacetic acid (TCA)-precipitable material. Two methods were devised for these determinations. The first

method involved the simultaneous hypodermic injection of ^{14}C-amino acid (^{14}C) and the bacterial isolate into the intercellular spaces of a pepper leaf. Bacteria were removed from the leaf at intervals after inoculation and analyzed for protein. Protein synthesis declined slowly over a 5-hr period in the incompatible interaction. Protein synthesis increased slightly over the same period in the compatible interaction of XV70-3 and 10-R. Protein synthesis declined slowly over the same period at similar rates in both XV69-20 and XV70-3 in the compatible interactions with ECW. The second method involved removal of bacteria from the various host-parasite interactions at specific intervals after inoculation, then the bacteria were exposed for 5 min to the ^{14}C-amino acid mixture (pulse-label). Protein synthesis in XV69-20 and XV70-3 after removal from 10-R remained essentially constant over the 5-hr period. However, protein synthesis increased in XV69-20 and XV70-3 after removal from ECW over a 24-hr period. Similar slight increases in "intracellular protein" and "total intracellular radioactivity" for each isolate were detected in 10-R over a 4.5-hr period.

Protein synthesis patterns were compared to two elements, electrolyte leakage and TCA-soluble radioactivity (presumably free labeled amino acids available to the isolates in the intercellular spaces). Increases in electrolyte leakage were associated with increases in protein synthesis over a 24-hr period during interactions of both isolates with ECW. In the incompatible interaction of XV69-20 and 10-R increases in electrolyte leakage were associated with increases in TCA-soluble radioactivity, but no increase in protein synthesis was detected in XV69-20.

Chloramphenicol and streptomycin sulfate inhibited protein synthesis almost entirely within 10 to 20 min after their addition to nutrient broth

cultures of XV69-20. In vivo, net protein synthesis was determined at specific intervals after hypodermic injection of XV69-20, ^{14}C, and one of the antimicrobial agents into 10-R leaves. Streptomycin was relatively ineffective in halting protein synthesis in XV69-20. Chloramphenicol may influence protein synthesis in XV69-20 because the incompatible interaction was affected as evidenced by the delay in onset of HR.

Considering the differential effect of chloramphenicol and streptomycin on protein synthesis in vivo, the similar kinetics of inhibition of protein synthesis in vitro, and other pertinent studies, it was postulated that the relative ineffectiveness of streptomycin in the determination of the induction period was attributable to an alteration of its bactericidal potential by the host. The use of some antimicrobial agents to determine an induction period in particular host-parasite systems may be inappropriate; therefore, a reevaluation of the three-phase concept of the bacterial-induced hypersensitive response would be indicated.

INTRODUCTION

Hypersensitivity, according to Muller (41), encompasses "all morphological and histological changes that, when produced by an infectious agent, elicit the premature dying-off (necrosis) of the infected tissue as well as inactivation and localization of the infectious agent." The hypersensitive response of higher plants to certain plant pathogens is a "defense reaction of plants against pathogens that occurs in an incompatible host-parasite relationship." (34)

The hypersensitive response (HR) is an intriguing topic for study by phytopathologists for several reasons (13). When plant hosts are inoculated with high numbers of phytopathogenic bacteria, large necrotic areas develop in leaves inoculated with incompatible types. Specificity is usually determined early in the incompatible reaction. Parasitic development is tissue undergoing HR is inhibited or stopped. The HR is "one of the earliest known events in incompatible reactions" (13); and therefore, may explain the specificity of pathogenicity.

Bacteria, fungi, insects, nematodes, and viruses induce HR in combination with certain hosts and non-hosts alike. All interacting systems with an associated HR have certain characteristics in common: inhibition of the pathogen, as well as collapse and necrosis of host cells. However, the bacterial-induced HR has been divided into three events termed the induction, latent, and tissue collapse phases (32).

The induction phase was defined by Klement (32) as " that period which is necessary for the bacterium to initiate the HR." The latent

phase is the time period from the end of the induction phase to the onset of a detectable response by the host. Hence, during the latent phase there is no sign of an adverse effect of the parasite on the host, although respiration of inoculated tissue increases. The third phase begins at the end of the latent phase and is characterized by the collapse of plant tissues.

The host-parasite system Capsicum annuum-Xanthomonas vesicatoria has been used extensively in research on hypersensitivity in plants (6, 8, 9, 58). It comprises near-isogenic lines of the host and races of the parasite. Since HR is usually associated with single-gene (race-specific) resistance (42), this system probably fits Flor's (18) gene-for-gene system. A quadratic check cannot be performed with this system because the appropriate genetic crosses cannot be made with bacteria. Evidence that X. vesicatoria does have a single gene mediating specificity comes from fluctuation analysis experiments by Dahlbeck and Stall (11). Avirulence is dominant to virulence when cells of both types are placed in pepper leaves simultaneously (55). Therefore, the C. annuum-X. vesicatoria system lends itself well to studies in specificity of host-parasite interaction when one considers the benefits of near-isogenic lines as presented by Daly (12).

This research was designed to test two hypotheses. First, the relative effectiveness of an antimicrobial agent in determination of induction periods for the bacterial-induced HR is correlated with the rate at which it inhibits protein synthesis in the bacterium in vivo, yet is not necessarily correlated with inhibition of protein synthesis in vitro. Second, cells of X. vesicatoria are metabolically stimulated in an incompatible reaction more than in a compatible reaction, and this stimulation can be

reflected by an increase in total bacterial protein or by an increased rate of protein synthesis by the bacterium.

REVIEW OF LITERATURE

The literature on the bacterial-induced hypersensitive response is voluminous. Meadows (39) presented a review of the bacterial-induced HR with emphasis on the induction period in 1976. Some new information on the induction period has been published since that time.

The induction period concept was advanced by Klement and Goodman (34). An induction period was first determined with the Nicotiana tabacum-Erwinia amylovora system and the N. tabacum-Pseudomonas syringae system. Klement and Goodman (35) injected a 500 mcg/ml solution of streptomycin sulfate to inhibit the bacteria at various times after inoculation of the tobacco with either bacterium. An injection of streptomycin 20 min or more after inoculation of tobacco with bacteria failed to prevent HR. It was concluded that only 20 min were needed for the bacteria to induce HR, and from that point the host was the active participant in the interaction, i.e., "certain host cell reactions complete the process" (35).

Klement (32) described two additional phases of the bacterial-induced HR: the latent phase and the period of tissue collapse. Using the N. tabacum-Pseudomonas phaseolicola system, Klement (32) determined an induction period of 3 to 4 hr. This estimate of the induction period was obtained with a different host-parasite combination and with a streptomycin sulfate concentration of 1000 mcg/ml. The induction period was dependent on the "physiological condition" of P. phaseolicola, and was not influenced by the host. An injection of streptomycin into leaves during the induction period prevented HR.

The latent phase of HR begins at the end of the induction period. This phase ends when the first signs of tissue collapse are noted. It was postulated that living bacteria were not necessary during the latent phase since injection of streptomycin during the latent phase failed to stop HR.

Klement (32) monitored changes in electrical conductivity during the progress of HR as an indicator of changes in permeability of the plasmalemma of the host cell. Conductivity measurements increased during the latent phase and reached a peak at the onset of tissue collapse.

Sule and Klement (60) concluded that a temperature of 37 C affected HR during the first two hours of the latent period. They postulated that certain host cell processes were thermosensitive. Meadows (39) obtained evidence contrary to their conclusion. With the C. annuum-X. vesicatoria system no HR occurred when leaves were placed at 39 C (temperature that inhibits X. vesicatoria) after inoculation. However, when pepper leaves inoculated with Pseudomonas marginata were placed at 39 C after inoculation, a HR did occur. P. marginata is able to grow at 41 C, but X. vesicatoria does not. It was concluded that the high temperature affected primarily the bacterium, and therefore affects the induction of HR by the bacterium. Durbin and Klement (16) reached the same conclusion after a reevaluation of Sule and Klement's previous work.

Meadows (39) and Meadows and Stall (40) obtained a wide variety of induction periods for the C. annuum-X. vesicatoria system by using a wide variety of antimicrobial agents. These agents differed in their modes of action on bacteria. Agents such as chloramphenicol provided much longer induction periods than did streptomycin sulfate. One of the least effective materials for inhibition of X. vesicatoria in pepper leaves was

streptomycin. During the experiments with chloramphenicol and streptomycin, cell collapse began 3 hr after inoculation of control leaves. Streptomycin prevented HR when added to inocula of X. vesicatoria, but it did not prevent HR when injected into leaves 1 hr after inoculation. Therefore, the induction period was less than 1 hr. Chloramphenicol prevented HR when added to inocula as well as when injected into leaves at 1 hr and 2 hr after inoculation with bacteria. Therefore, the induction period obtained with chloramphenicol was at least 2 hr long.

Meadows (39) also determined the induction period for the C. annuum-X. vesicatoria system by placing detached leaves at 39 C at specific time intervals after inoculation. Cell collapse began in control leaves at 5 hr after inoculation. The induction period was at least 3 hr because leaves incubated at 30 C for more than 3 hr before transfer to a temperature of 39 C responded in a hypersensitive manner.

Sequeira (52) reported a 3 hr induction period for the N. tabacum-Pseudomonas solanacearum system. Apparently, rifampicin and streptomycin were equally effective in its determination. Lyon and Wood (37) reported a 60 to 90 min induction period for the Phaseolus vulgaris-Pseudomonas mors-prunorum system by injection of either streptomycin (55 mcg/ml) or chloramphenicol (100 mcg/ml). A 90 to 105 min induction period was determined by Durbin and Klement (16) for the N. tabacum-P. syringae system by infiltrating leaves with 1000 mcg/ml solution of streptomycin. Gulyas et al. (25) reported induction periods for 3 systems. The time for induction for each system N. tabacum-Pseudomonas pisi, N. tabacum-P. phaseolicola, and N. tabacum-X. vesicatoria was 1.5 hr, 3-4 hr, and 5-6 hr, respectively.

The injection of streptomycin appears to be an acceptable and somewhat standard practice to determine the induction period. Different induction periods are derived from different host-parasite combinations. Sequeira (52) noted that induction periods vary with the species and the population of the bacterium used. Unfortunately, one aspect which seems to have been ignored in determinations of the induction period is the effect of the host on the antimicrobial agent. This aspect could be crucial when one considers the behavior of streptomycin once it enters certain plant tissues (22). The converse of this aspect, the effect of the antimicrobial agent on the host, has been considered in at least two reports (40, 49).

The C. annuum-X. vesicatoria system is a highly specific host-parasite system. The origin of the resistance in pepper was P. I. 163192, which Sowell (54) had observed to be resistant in field and greenhouse testing to an isolate of X. vesicatoria obtained from tomatoes in Bradenton, Florida. Cook and Stall (5) discovered in 1963 that the factor in plants of P. I. 163192 which conferred resistance to X. vesicatoria was simply inherited and dominant. Then, Stall and Cook (56) determined that the observed resistance was characterized by a hypersensitive response.

Races of X. vesicatoria were classified by Cook and Stall (7) in 1969 according to the responses induced in tomato and in genetically different pepper lines. Isolates of X. vesicatoria which produced a hypersensitive response in pepper and a susceptible response (SR) in tomato were designated to be the "tomato strain" of X. vesicatoria. Those that were pathogenic in tomato and pepper were named the "pepper strain" of X. vesicatoria. Pepper lines containing the dominant gene derived from P. I. 163192 in the homozygous state were hypersensitive to

certain isolates of the pepper strain. These isolates were called race 2, pepper strain. Isolates of X. vesicatoria which could only induce a SR in breeding lines homozygous for the dominant gene were designated to be race 1, pepper strain.

The mutation of race 2, pepper strain to race 1, pepper strain occurred at a rate of 4×10^{-4} per cell per division in fluctuation analysis experiments by Dahlbeck and Stall (11). Mutation of the tomato strain to race 2, pepper strain occurred at a similar rate. But mutations of the tomato strain to race 1, pepper strain were not detected. It was postulated that the latter change would require mutation of two genes. Thus, a change in one gene caused a change in race specificity.

Ercolani (17) proposed in 1970 a "unified hypothesis of induced resistance against bacterial infection" to explain specificity and triggering of HR. Although it was based on data obtained with the Lycopersicon esculentum—Corynebacterium michiganese system, Ercolani suggested that it may be applicable to other systems. Hence, the HR of the C. annuum-X. vesicatoria system may be explained by this hypothesis. According to Ercolani's hypothesis, complementary sites exist on the host and the bacterial cell. The host cell has transit sites and multiplication sites. Regardless of their specificity, all bacterial cells are able to interact with transit sites, yet "bacterial activity" is unaffected. Multiplication sites, however, are suitable sites for "bacterial activity", that is, certain bacteria are able to attach to these sites and to multiply there. The sensitivity locus and the susceptibility locus are present at the multiplication site.

Certain bacteria are able to activate the sensitivity locus. Upon activation, certain stimuli are transmitted to bacteria, which influence

their metabolic activity and production of "factors controlling the HR." Specific bacteria (those which are virulent in a particular host) activate the susceptibility locus. After activation of this locus, the hypersensitive-reaction-inducing factors (HRI) are negated, and a susceptible plant response occurs. An HR occurs when certain bacteria activate the sensitivity locus but the not susceptibility locus. The bacterium possesses a virulence locus and a pathogenicity locus, which correspond, respectively, to the plant's sensitivity locus and susceptibility locus. It is the interaction of these two pairs of loci that influences the host plant's response.

Sequeira (52) stated that Ercolani's hypothesis on the induction of HR appears to be supported by existing evidence. Ercolani's hypothesis was explained by Sequeira (52) as follows: "1. incompatible bacteria attach to an infectible site on the host cell wall, 2. that, as a result, bacteria become metabolically activated and release a highly unstable inducing factor (HRI), and 3. that this substance interacts with a 'sensitivity locus' on the host cell, resulting in the release of the factor (HRF) that causes the HR. The HRF is ultimately responsible for the collapse of the host cell and causes a rapid reduction in bacterial populations."

It might be inferred from Sequeira that only incompatible bacteria are "metabolically activated." However, Ercolani proposed that both compatible and incompatible bacteria are activated when the sensitivity locus is triggered. No mention was made as to a relative degree of stimulation of the compatible and incompatible bacteria. Sequeira's interpretation of Ercolani's hypothesis differs somewhat from Ercolani's (17) original writings. Sequeira stated that the HRI was released by the

bacterium. Ercolani indicated that the HRI was released by the host. Apparently, Sequeira used HRF to refer to Ercolani's HRI; yet, he has also stated that the HRI and HRF may or may not be separate factors.

In 1978, Sasser (49) presented convincing evidence that protein synthesis was an essential process for induction of HR. The two antimicrobial agents chloramphenicol and streptomycin were used by Sasser (49) to determine if bacterial protein synthesis was involved in the induction of HR rather than to determine the length of the induction period. In the N. tabacum-P. pisi system, HR was prevented when each antimicrobial agent was injected simultaneously into tobacco leaves with P. pisi. His^- and Arg^- amino acid auxotrophs of P. pisi did not induce HR in tobacco unless the deficient amino acid was added to the inoculum. However, he did not establish any connection between the probable existence of a HRI and the possibility that it could be proteinaceous in composition. Sasser has asserted that the relationship of protein synthesis to HR may be indirect.

Evidence for the involvement of extracellular protein(s) in the bacterial-induced HR was obtained by Sasser (50) in 1980. A variety of proteases, when added to P. pisi and X. vesicatoria inoculum, inhibited the development of HR in tobacco and cowpea. The inhibition of HR was reversed when the plants were treated with a protease inhibitor.

This research was designed to test two hypotheses. First, the relative effectiveness of an antimicrobial agent in determination of induction periods for bacterial-induced HR is correlated with the rate at which it inhibits protein synthesis in the bacterium in vivo, yet is not necessarily correlated with inhibition of protein synthesis in vitro. Second, cells of X. vesicatoria are metabolically stimulated in an incompatible reaction more than in a compatible reaction, and this stimulation can be

reflected by an increase in total bacterial protein or by an increased rate of protein synthesis by the bacterium.

MATERIALS AND METHODS

Test Plants

Two selections of pepper, Capsicum annuum L., were used in this work. The cultivar 'Early Calwonder' (ECW) was a susceptible host, and the experimental line, 10-R, was both a hypersensitive and a susceptible host, depending on the strain of the parasite. The 10-R host has a gene from P. I. 163192 (56) that conditions a hypersensitive response (HR) to certain strains of the parasite under study, and is near isogenic to ECW.

The host plants were maintained in steamed soil in a large greenhouse bench. A 6-6-6 commercial fertilizer was added periodically to bench soil to maintain actively growing plants. Fertilizer was applied at a rate of about 280 kg/ha.

Bacteria

Two isolates of Xanthomonas vesicatoria (Doidge) Dows. (XV) were used in the experiments. Isolate XV69-20 was obtained in 1969 from diseased commercial pepper plants growing at Delray Beach, Florida, and caused an HR when injected into leaves of 10-R and a susceptible response (SR) in ECW. Isolate XV70-3 was obtained in 1970 from the same host in the same area of Florida, and resulted in a SR in leaves of both peppers.

Stock cultures of XV69-20 and XV70-3 were maintained in nutrient broth at 5 C. As cultures were needed, portions were removed from the stock flasks and added to nutrient broth for growth. Cultures were then incubated overnight at 30 C on a rotating shaker. Stock cultures were replaced periodically to insure the purity of each strain. Passage of

both XV69-20 and XV70-3 through 10-R and subsequent reisolation of the bacteria from leaves with symptoms of HR and SR, respectively, were followed by single colony selection for new stock cultures. Once established, new stocks were checked for disease reaction in 10-R.

Preparation of Inocula

Logarithmically growing cultures of X. vesicatoria were centrifuged for 10 min at 1800 g. The supernatant was discarded and the pellet was resuspended in deionized water. The suspensions were adjusted to a desired optical density (OD) at a wavelength of 600 nm using a Spectronic 20 colorimeter, and were then placed in beakers on a slowly rotating shaker during use.

Inoculations

Inoculations were accomplished by injection-infiltration of leaves (31). Inoculations with radioactive cells were performed with excised leaves in a shield (Fig. 1 and 2). Petioles of the excised leaves were inserted through a hole in parafilm that covered a small test tube containing water before placement in the shield. A suspension of radioactive bacteria was poured from a test tube into the syringe, the syringe plunger replaced, and the excess air expelled from the syringe inside the shield. After inoculation, excess inoculum was removed from the leaf surface with cheesecloth before removal of the leaf from the shield.

Leaves were inoculated with radioactive bacteria while on the plant in some experiments. This was accomplished by enclosing the leaves in a Ziploc^{R} sandwich bag (The Dow Chemical Company, P. O. Box 68511, Indianapolis, Indiana 46268) and closing the end of the bag around the petiole. Inocula were placed in the syringe as before. Injection of the enclosed leaves was made by inserting the syringe needle through the Ziploc^{R} bag

Fig. 1. Shield used when excised leaves were injected with bacterial suspensions containing radioisotope and when radioactive suspensions were transferred from test tubes to syringes.

Fig. 2. Simulation of the method of injection within the shield. The leaf petiole was contained in a test tube partially filled with tap water during actual injections in experiments.

and into the abaxial leaf surface. Excess inoculum collected in the bottom of the bag. A large shield was placed in back of and to each side of the plant to avoid any spread of radioactive contamination if an accident occurred during injection. A plastic bag was wrapped around the clay pot and was sealed around the plant stem with label tape. After all leaves were infiltrated, each bag was carefully taken from each leaf. Excess inoculum was removed from each leaf with cheesecloth. After the experiment was completed, the plant was decapitated, and the roots, soil, and clay pot were returned to the greenhouse.

Half-leaf Method

Potential variation from leaf to leaf on each plant was circumvented by using a half-leaf experimental design. Most experiments involved two treatments, i. e., a comparison of two strains of bacteria or a comparison of a treatment and a control. The half-leaf treatments were separated by the midvein of the leaf. One leaf served as one replicate.

Leaf Excision Technique

Excised leaves were used in certain experiments. To reduce air embolisms, a drop of water was placed on the leaf petiole at the point where the leaf was severed with a sharp razor or scalpel blade. The petiole of the excised leaf was placed immediately into a test tube containing water.

Incubation of Inoculated Test Plants

Incubations occurred in two types of controlled environment chambers. The first chamber, the Sherer Controlled Environment Chamber (Model CEL 4-4, Sherer-Gillett Company, Marshall, Michigan 49068), was kept at 30 C. The left and right compartments of this chamber had light sources that emitted 6300 and 7950 lux, respectively, at a distance of 75 cm from the

source. The light source was primarily fluorescent with a small amount of incandescent light. The other chamber was custom-made as shown in Fig. 3. It was constructed of cardboard and thin clear plastic. This chamber was placed on top of a waterbath adjusted to provide a chamber temperature of 30 C. Fluorescent lighting illuminated the chamber to an intensity of 5500 lux at a distance of 35 cm from the source. The entire apparatus was contained in a fume hood which was operated during the course of each experiment.

Incubation of Excised Leaves

Excised leaves remained water-soaked after inoculation unless special procedures were undertaken. Drying time was minimized by positioning the abaxial leaf surface upward (Fig. 4), exposing it to light, and maintaining air flow around each leaf.

Removal of Bacteria from Intercellular Spaces

Two methods for removal of bacteria from inoculated leaves have been described (26, 33), but two variations of these methods were devised for this work. In method A, leaves were first water-soaked in a shallow pan that contained enough deionized water to cover the leaves. A small weight was applied to keep the leaves submerged. The pan was placed in a chamber connected to a vacuum pump which was operated continuously for 6 min to maintain a vacuum of 27 to 28 mm Hg. Upon return of air to the chamber water was forced into intercellular spaces of each leaf. In order to obtain uniform water infiltration, it was necessary to repeat this process on occasion. Surface water was removed from the water-saturated leaves with cheesecloth, the midveins were removed with a sharp razor blade, and each half-leaf was rolled tightly from the end distal to the petiole to the end proximal to the petiole. The rolled half-leaf was

Fig. 3. Custom-made growth chamber in the fume hood. The front flap was opened so that the port through which heated air entered was visible in the photograph.

Fig. 4. Illustration of the inverted position that minimized drying time of excised leaves which had been inoculated in the Sherer growth chamber.

inserted into a 16 x 100 mm test tube with the severed portion downward so the top of the rolled half-leaf was 6 mm above the rim of the test tube (Fig. 5). The half-leaf unfurled so that maximal contact was obtained between the adaxial leaf surface and the test tube wall. This maximal contact was critical so that the leaf did not fall to the bottom of the test tube during centrifugation. Large leaves were trimmed to fit into the tube to avoid damage to the leaf. The test tubes containing the half-leaves were placed in an angle centrifuge which was operated at 1800 g for 4 min. Then, the half-leaf was removed from each test tube, and the remaining suspension was agitated in each tube on a Vortex mixer. In method B, the midveins were excised from leaves as before. Each half-leaf was rolled and inserted into an individual test tube so that the top of the half-leaf fell 6 to 12 mm below the rim. Deionized water or a deionized water solution of an appropriate antimicrobial agent at a concentration of 500 mcg/ml was added to the test tubes containing half-leaves. This measure was taken to prevent dilution of the agent injected initially with the bacteria. The tubes were placed in a vacuum of 27 to 28 mm Hg for 6 min and then removed. After discarding the fluid in each test tube, each tube was centrifuged at a very low speed for about 20 sec to remove any superficial moisture from the half-leaf's surface. This 1 to 2 ml of fluid was taken from each tube with an elongated Pasteur pipette inserted through the center of the rolled half-leaf. After centrifugation at 1800 g for 4 min, the half-leaves were discarded.

Determination of Bacterial Concentrations

Colony-forming Units

A duplicate series of ten-fold dilutions was made from each sample of a bacterial suspension. A 0.05-ml portion of the bacterial suspensions

Fig. 5. (left) Position of rolled half-leaves in test tubes prior to centrifugation. (right) Position of half-leaves in the test tubes after centrifugation when the bacterial suspension had been separated.

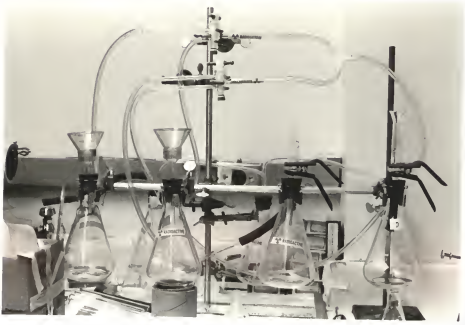

Fig. 6. The bank of filtration devices which facilitated rapid filtration of numerous samples. A respirator was used to maintain the partial vacuum to promote movement through the filter.

was transferred to 0.45 ml of sterile tap water in 16 x 100 mm test tubes with capillary transfer pipettes (Cooke Laboratory Products, 900 Slaters Lane, Alexandria, Virginia 22314). A series of 1 in 10 dilutions was made from each suspension. Contents of the last 2 or 3 tubes in a dilution series were poured onto separate nutrient agar plates which had dried at 35 C for 5 days; plates were then incubated at 30 C for 3 to 5 days.

Essentially, the same procedure was followed with bacteria labeled with ^{14}C, except that serial dilutions were made in 10 x 75 mm disposable plastic petri plates, and incubated at room temperature.

Petroff-Hauser Method

A viable count method was not possible for monitoring cell numbers in the presence of streptomycin sulfate, because it binds irreversibly to bacterial ribosomes. Therefore, a direct count method was used. Bacterial suspensions of undetermined concentrations were diluted with glycerol. The glycerol functioned to slow down bacterial movement, which permitted more accurate counting. The glycerol also served to dilute highly-concentrated samples. Rod-shaped particles were counted under a 40X phase-contrast microscope lens. The numbers in 40 to 50 of the 0.05 mm x 0.05 mm squares in a Petroff-Hausser Bacteria Counter (Hausser Scientific, Township Line Road at Blue Bell Road, Blue Bell, Pennsylvania 19422) were determined for each sample. This was sometimes sufficient for a 0.95 confidence coefficient. Duplicate counts were made of all samples.

Conductivity Measurements

A Model 31 Conductivity Bridge (Yellow Springs Instrument Company, Inc., Yellow Springs, Ohio 45387) was used for measuring electrolytes

leaking from pepper cells. Leaf discs, either 10 or 15 mm in diam, were cut with a Number 9 or 11 cork borer, respectively. Discs from each half-leaf were placed in a separate 50-ml beaker containing 15 ml of deionized water, and were kept submerged with a nylon screen. When all nylon screens were in place, a conductivity reading was recorded for each sample. The discs were then placed in a vacuum of 25 mm Hg for 3 min which resulted in water-soaking of the discs upon release of the vacuum. Afterward, the beakers were placed for 1 hr on a shaker that was reciprocating at 120 cycles/min. Then, a final conductivity reading was taken. Net conductivity was determined by the difference between the initial and the final readings. An increase in conductivity indicated leakage of electrolytes.

Determination of Protein Synthesis and TCA-soluble Radioactivity

The proteins in bacterial samples which had been exposed to a ^{14}C-amino acid mixture were precipitated by adding 1 ml of ice cold 5% trichloroacetic acid (TCA) to 0.04 ml of the bacterial suspension. Then, the samples were maintained at 90 C for 20 min in a water bath to release aminoacyl-transfer RNAs through hydrolysis of the cis-glycol amino acid ester bonds (4). When tubes had cooled to room temperature, their contents were agitated on a Vortex mixer, and were added to separate GN-6 membrane filters (0.45 micron, 13 mm diam) (Gelman Instrument Company, Ann Arbor, Michigan 48106) which had been premoistened with 1.0 ml of 5% TCA. Each tube was rinsed twice with 3.8 ml of 5% TCA, and the rinsing fluid was also poured through the membrane filter. The GN-6 membrane filter and the filter funnel were rinsed again with 15 ml of 5% TCA from a squeeze bottle. In experiments in which TCA-soluble radioactivity

was determined. the entire filtrate (approx 24.6 ml) was collected in a large test tube contained in the large Erlenmeyer flasks of the filtration device. After filtering, the tube's contents were shaken by inverting the test tube several times (with a parafilm lid on the tube), and 1.0 ml of the filtrate was added to 10.0 ml of Aquasol-2^{R} cocktail. (The bank of filtration devices shown in Fig. 6 facilitated the filtration process.)

All filters from an experiment were washed for 8 hr in a common bath consisting of 600 ml of 5% TCA. Filter discs were kept in motion with a magnetic stirring bar. Taken from the common bath, the filters were put on Kimwipes^{R}, which absorbed any excess bathing solution. The filters were then ready for placement in the appropriate liquid scintillation cocktail.

Radioactivity was determined by counting with a Beckman LS-150 Liquid Scintillation System (Beckman Instruments, Inc., Campus Drive at Jamboree Boulevard, P. O. Box C-19600, Irvine, California 92173). The ^{14}C-amino acid (^{14}C) beta particles were counted in a wide ^{14}C window with the automatic quench compensation (AQC) function in operation. Counting of each sample was terminated when 2 σ counting error was reached.

Net Synthesis and Rate of Synthesis of Protein

Bacterial suspensions mixed with a ^{14}C-amino acid mixture were removed from pepper leaves at specific time intervals after inoculated leaves appeared to be dry. The suspensions were immediately agitated on a Vortex mixer and 0.04 ml of the suspension was added to 1.0 ml of ice cold 5% TCA. The net amount of protein synthesis was determined from counts of radioactivity.

Bacterial samples obtained from either inoculated leaves or cultures were pulse-labeled to measure the rate of protein synthesis. Exactly 0.04 ml of a bacterial sample was put into a 12 x 75 mm test tube containing 0.002 ml (0.2 microCurie) of ^{14}C. Each test tube was placed in a 30 C Warburg apparatus which had been converted to a waterbath shaker as illustrated in Fig. 7 and 8. The shaker was oscillating at 100 cycles per min. The bacteria remained in the mixture for 5 min, then 1.0 ml of ice cold 5% TCA was added to each test tube. This process was repeated at progressive times to obtain a rate of protein synthesis.

Preparation of Blanks

Experiments which involved pulse-labeling of bacterial cells in test tubes could be analyzed only if a blank was prepared for each individual sample. Blanks were prepared according to method used by Glaser (21). A 0.04-ml portion of bacterial suspension was added to a 10 x 75 mm test tube containing 0.002 ml (0.2 microCurie) of the ^{14}C-amino acid mixture and 1.0 ml of ice cold 5% TCA. Blanks were then processed in the same manner as the pulsed samples. Sample and blank membrane filters were washed together in the final common bath.

Preparation of blanks for experiments that did not involve the pulse-label method was more intricate. These experiments involved the centrifugation of labeled bacteria from half-leaves, followed by removal of 0.04 ml of bacterial suspension for subsequent extraction with TCA. The remainder of the suspension was filtered through a GA-6 membrane filter (0.45 micron, 13 mm diam) (Gelman Instrument Company) using a Swinney syringe filter holder (Gelman Instrument Company). Then, 0.04 ml of the filtrate was added to a test tube containing 1.0 ml of ice cold 5% TCA. Blanks were processed in the same manner as samples analyzed for protein.

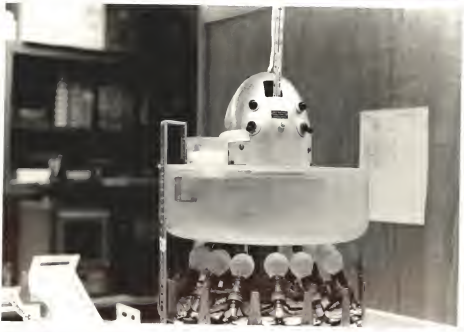

Fig. 7. Modification of a Warburg apparatus to a constant temperature waterbath shaker.

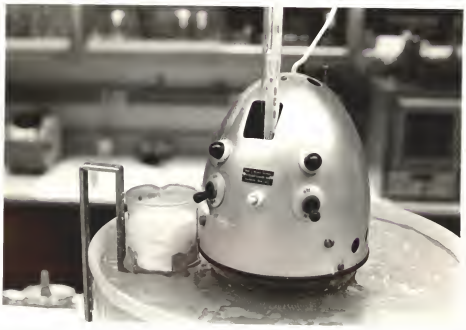

Fig. 8. Illustration of the beaker in the waterbath shaker which contained styrofoam with holes to accommodate several 12 x 75 mm test tubes. About 3/4-inch of water filled the beaker to promote transfer of heat to each test tube.

LSC Cocktails and Membrane Filters

Two cocktails and two types of membrane filters were used. Aquasol-2® (New England Nuclear, 549 Albany Street, Boston, Massachusetts 02118) dissolved large quantities of water, yet provided a high counting efficiency. This cocktail was used alone or in combination with GA-6 membrane filters (cellulose triacetate, 0.45 micron pore size, 25 mm diam) (Gelman Instrument Company). These filters were transparent in Aquasol-2®. This cocktail was used to analyze radioactive filtrates composed largely of 5% TCA. The ratio of filtrate (TCA) to cocktail was calculated to provide either a clear solution or a transparent gel because a two-phase mixture can occur when cocktail-water ratios are not considered.

Ready-Solv® HP cocktail (Beckman Instruments, Inc.) was used in combination with GN-6 membrane filters (cellulose nitrate, 0.45 micron pore size, 25 mm diam). Ready-Solv® "solubilized" these filters as well as biological samples. This cocktail dissolved much less water than did Aquasol-2®. This combination of cocktail and membrane filter was used to analyze radioactivity in TCA-extracted samples. It was thought that both extracted sample and filter paper could be "solubilized" completely with this cocktail.

Dispensation of Isotope

An isotope dose was delivered with either a 0.1, 0.25, or 0.5 ml gas-tight Hamilton syringe (Hamilton Company, P. O. Box 10030, Reno, Nevada 89510) that was fitted with a 22-gauge or 26-gauge 5-cm needle. In the pulse-labeling experiments, a 0.1 ml syringe was used to deliver 0.002 ml (0.2 microCurie) of ^{14}C-amino acid mixture to the bottom of a 12 x 75 mm test tube. The syringes of 0.25 and 0.5 ml size were used in

net protein synthesis experiments to deliver 0.01 ml (1.0 microCurie) of ^{14}C mixture to a 10 x 75 mm or a 12 x 75 mm test tube containing 2.0 ml of bacterial suspension. The ratio of the volume of bacterial suspension to the amount of isotope was 20:1 or 200:1 in experiments on the rate of protein synthesis or in experiments on net protein synthesis, respectively. A model PB600 Hamilton dispenser was used to insure that treatments and replicates of treatments received equal doses of radioisotope.

Antimicrobial Agents and Chemicals

Chloramphenicol was obtained from Sigma Chemical Company, P. O. Box 14508, St. Louis, Missouri 63178. Streptomycin sulfate was purchased from ICN Pharmaceuticals, Inc., Cleveland, Ohio 44128. Glycerol and TCA were available from Mallinckrodt, Inc., Paris, Kentucky 40361. Absolute ethanol was obtained from Mallinckrodt, Inc., St. Louis, Missouri 63147; and nutrient broth and agar were available from Difco Laboratories, Detroit, Michigan 48232. Some nutrient broth came for BBL, Cockeysville, Maryland 21030. The (U-^{14}-C)-L-amino acid mixture (100 microCuries/ml; 268 mCi/mmole) was purchased from ICN Chemical and Radioisotope Division, 2727 Campus Drive, Irvine, California 92715; and (U-^{14}-C)-L-amino acid mixture (100 microCuries/ml; 298 mCi/mmole) was obtained from New England Nuclear, 549 Albany Street, Boston, Massachusetts 02118.

RESULTS

Relationship Between Optical Density and Colony-forming Units

An optical density, $OD_{600\ nm}$, of 0.3 for deionized water suspensions of some isolates of Xanthomonas vesicatoria corresponded to a concentration of approx 1.0×10^8 colony-forming units (cfu)/ml (58). The relationship of optical density and cfu/ml was determined for isolates of X. vesicatoria used in this investigation.

Log phase cultures of XV69-20 and XV70-3 were pelleted and suspended in deionized water. Six different optical densities from a range of 0.07 to 0.70 were prepared. The cfu/ml in each OD was determined by dilution-plating. Regression lines were determined from the data (Fig. 9). The numbers of cells of XV69-20 and XV70-3 at a $OD_{600\ nm}$ of 0.3 were approx 6.0×10^8 cfu/ml and 4.1×10^8 cfu/ml, respectively.

The concentration of both strains at different optical densities in nutrient broth was also determined. Nutrient broth absorbs primarily at a wavelength of 400 nm (30), but the relationship between cfu and OD at 600 nm was about the same for each bacterium in nutrient broth and in deionized water (Fig. 9).

Comparison of Rates of Growth and Protein Synthesis in XV69-20 and XV70-3

XV69-20 (pepper strain, race 2) and XV70-3 (pepper strain, race 1) differ in the characteristic of avirulence to 10-R pepper leaves. The rates of growth and protein synthesis of these bacteria in nutrient broth cultures were compared. Log phase cultures of XV69-20 and XV70-3 at equal optical densities were adjusted to a concentration of approx

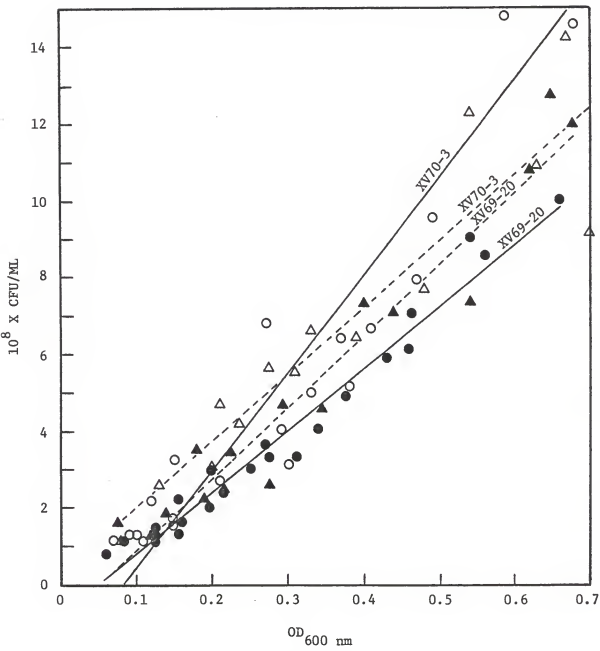

Fig. 9. The relationship between optical density (OD) and colony-forming-units (CFU) for deionized water suspensions (broken lines) of XV69-20 (▲) and XV70-3 (△), and for nutrient broth suspensions (solid lines) of XV69-20 (●) and XV70-3 (○). Regression line equations for deionized water suspensions are y=-0.89 + 18.56x for XV69-20 and y=0.39 + 17.18x for XV70-3. Regression line equations for nutrient broth suspensions are y=-0.74 +16.03x for XV69-20 and y=-2.00 + 25.35x for XV70-3.

1 x 10^8 cfu/ml in nutrient broth in a 125-ml sidearm flask. The OD was measured, a 0.05-ml sample was removed for dilution-plating, and a 0.04-ml sample was taken for analysis of protein synthesis every half-hour for a 3-hour period. After this 3-hour period, measurements and samples were taken hourly until the seventh hour of incubation. Protein synthesis was determined by the pulse-labeling technique. Regression lines were calculated for growth and protein synthesis rates.

Based on turbidity measurements and cfu counts, XV69-20 and XV70-3 grew at equivalent rates (Fig. 10). The rates of protein synthesis of the isolates were also equivalent (Fig. 11). The regression coefficients in each pair of regression lines were similar.

Net Protein Synthesis in X. vesicatoria and TCA-soluble Radioactivity of Filtrates in Compatible and Incompatible Interactions

Protein synthesis of the two strains of X. vesicatoria in excised leaves of pepper plants was compared. Leaves of 10-R and ECW pepper plants were inoculated with each strain. This experiment was performed to determine if the rate of bacterial protein synthesis in the leaves could be related to HR development.

Nutrient broth cultures of XV69-20 and XV70-3 were adjusted by temperature control to obtain equal optical densities. The $OD_{600\ nm}$ of 0.3 corresponded to a concentration of approx 5.0 x 10^8 cells/ml for each culture. Two ml of the bacterial suspension were added to 10 x 75 mm test tubes and placed on a shaker reciprocating at 120 cycles/min. Immediately prior to injection of pepper leaves with bacteria, 0.01 ml (0.1 micro-Curie) of a ^{14}C-amino acid mixture was added to each tube. Using the half-leaf experimental design, each of the 24 leaves from 10-R or ECW plants was inoculated with XV69-20 and XV70-3. When visible signs of water-soaking disappeared, that point was designated as zero time. Pairs

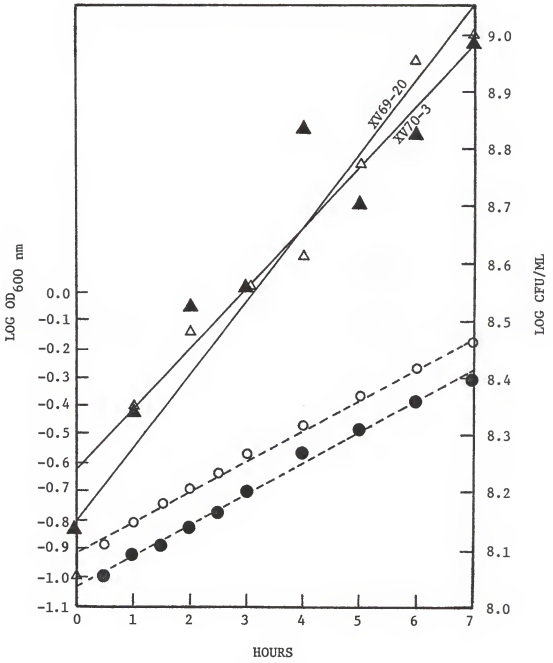

Fig. 10. Rates of bacterial growth in nutrient broth cultures determined by a dilution plate technique for XV69-20 (△) and XV70-3 (▲) or by a turbidimetric method for XV69-20 (○) and XV70-3 (●). Regression line equations for growth as determined by a dilution plate technique are y=8.1567 + 0.1259x for XV69-20 and y=8.2284 + 0.1091x for XV70-3. Regression line equations for growth as determined by a turbidimetric method are y=-0.91 + 0.107x and y=-1.04 + 0.109x for XV69-20 and XV70-3, respectively.

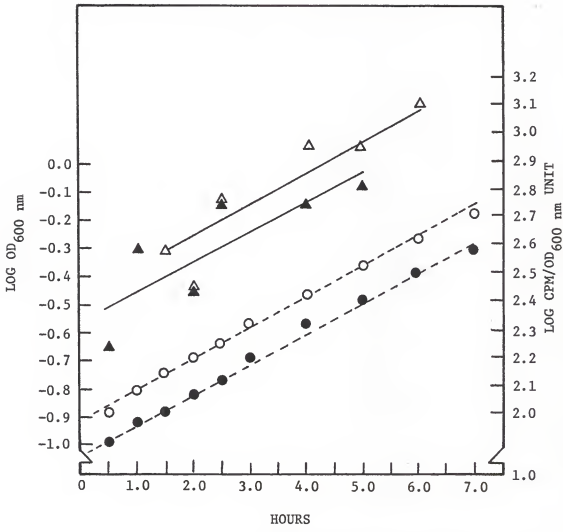

Fig. 11. Rates of protein synthesis in cultures of XV69-20 (△) and XV70-3 (▲) and the relationship of the rates of protein synthesis to the rates of growth in cultures of XV69-20 (○) and XV70-3 (●). Regression line equations for rates of protein synthesis are y=2.405 + 0.112x for XV69-20 and y=2.319 + 0.105x for XV70-3. Regression line equations for rates of growth are y=-0.91 + 0.107x for XV69-20 and y=-1.04 + 0.109x for XV70-3.

of leaves were removed for sampling at zero time and at hourly intervals, thereafter. One pair of these leaves was sampled for electrolyte leakage measurements, and net protein synthesis in bacteria was determined from another pair of leaves. Bacteria were removed from a leaf by Method A for protein synthesis determination, and protein was extracted from 0.04 ml of the bacterial suspension. The radioactivity was also determined in the TCA filtrates collected during the extraction process. The number of bacteria per ml in the suspensions removed from leaves was determined by dilution-plating.

Values obtained from samples taken from duplicate half-leaves were averaged. The average counts per minute (cpm) per 0.04 ml of bacterial suspension was converted to cpm/ml value. The cpm/ml value was divided by the cfu/ml value obtained for a particular sample. Therefore, protein synthesis was expressed as cpm/cfu or as the log (cpm/cfu). Linear regression equations were calculated for net protein synthesis by each bacterium in each host.

Net Protein Synthesis

The patterns of net protein synthesis by XV69-20 and XV70-3 in the ECW leaves were similar (Fig. 12). Slopes of regression lines were similar. The decreasing levels of net protein synthesis by XV69-20 and XV70-3 in the compatible interactions were associated with no change in electrolyte leakage as indicated by the regression lines calculated for electrical conductivity data. Regression coefficients for net protein synthesis by cells of XV69-20 and XV70-3 in the 10-R host (Fig. 13) were similar. However, the electrolyte leakage from leaves inoculated with XV69-20 was greater than the electrolyte leakage from leaves inoculated with XV70-3.

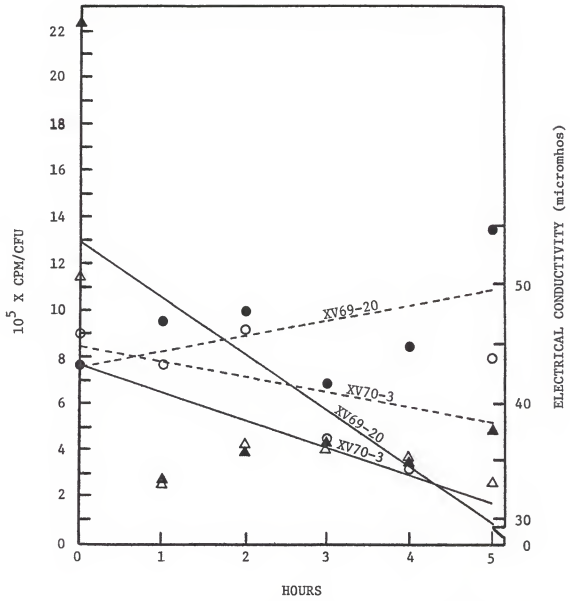

Fig. 12. Protein synthesis by XV69-20 (▲) and XV70-3 (△) in ECW pepper leaves. Conductivity measurements of ECW half-leaves inoculated with XV69-20 (●) and XV70-3 (○) are indicative of the progress of the susceptible response. Regression line equations for protein synthesis are y=0.000130 - 0.000024x for XV69-20 and y=0.000077 - 0.000012x for XV70-3. Regression line equations for protein synthesis are represented by solid lines, and regression line equations for conductivity measurements are represented by broken lines. Based on regression analysis, overall differences between XV69-20 and XV70-3 in terms of protein synthesis were not significant ($P > 0.05$).

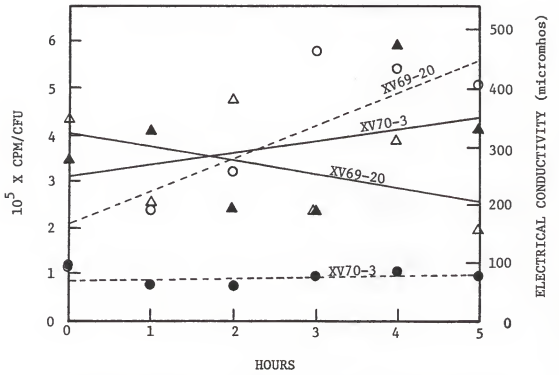

Fig. 13. Protein synthesis by XV69-20 (△) and XV70-3 (▲) in 10-R pepper leaves. Conductivity measurements of 10-R half-leaves inoculated with XV69-20 (○) and XV70-3 (●) are indicative of the progress of the hypersensitive and the susceptible responses. Regression line equations for protein synthesis are $y=0.000041 - 0.000003x$ for XV69-20 and $y=0.000031 + 0.000003x$ for XV70-3. Regression line equations for protein synthesis are represented by solid lines, and regression line equations for conductivity measurements are represented by broken lines. Based on regression analysis, overall differences between XV69-20 and XV70-3 in terms of protein synthesis were not significant ($P > 0.05$).

TCA-soluble Radioactivity

Counts of radioactivity in the 24.6 ml of filtrate were converted to cpm per 1.0 ml of original sample. In the compatible host-parasite combinations the amount of TCA-soluble radioactivity in 0.04 ml of sample decreased slowly over the 5-hour assay period (Fig. 14). However, the TCA-soluble radioactivity with the incompatible combination of XV69-20 and 10-R increased substantially (Fig. 15). This increase was paralleled by an increase in electrolyte leakage with the incompatible interaction.

Rates of Protein Synthesis by X. vesicatoria Removed from Compatible and Incompatible Interactions

Bacteria were removed from leaves during the host-parasite interaction to determine the rate of protein synthesis using the pulse-label technique. The experimental procedure was similar to that in the net protein synthesis experiments except that the isotope was not injected into the leaves with the inocula and the time course was extended to 24 hours with ECW as a host. The rates of protein synthesis of both isolates in ECW increased in a similar manner over the 24-hour period, and these increases in rate were paralleled by increases in electrolyte leakage caused by both isolates in ECW (Fig. 16). The similar rates of protein synthesis of the two isolates in 10-R did not change substantially over the 5-hour test period. An increase in electrolyte leakage noted for XV69-20 was not associated with any increase in its rate of protein synthesis in 10-R (Fig. 17).

Net Protein Synthesis and Radioactive Pools in Bacteria Removed from Intercellular Fluids

The procedure of extracting protein from suspensions of X. vesicatoria removed from pepper leaves to determine protein synthesis by X. vesicatoria possibly may have included radioactive proteins produced by

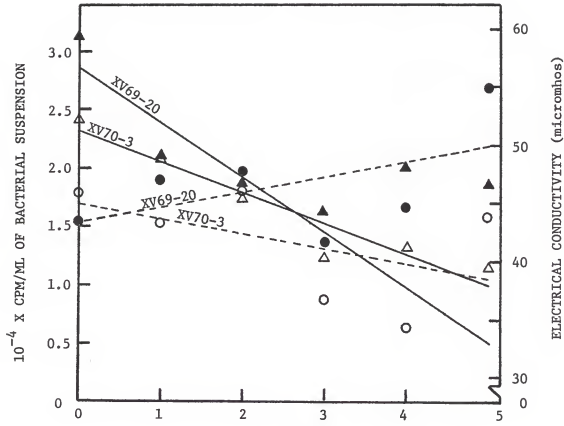

Fig. 14. Trichloracetic acid-soluble radioactivity in suspensions of XV69-20 (▲) and XV70-3 (△) removed from the intercellular spaces of half-leaves of ECW pepper plants. Conductivity measurements of ECW half-leaves inoculated with XV69-20 (●) and XV70-3 (○) are indicative of the progress of the hypersensitive and the susceptible responses. Regression line equations for TCA-soluble radioactivity are $y=25,987 - 2,006x$ for XV69-20 and $y=23,222 - 2,670x$ for XV70-3. Regression line equations for TCA-soluble radioactivity are represented by solid lines, and regression line equations for conductivity measurements are represented by broken lines. Based on regression analysis, overall differences between XV69-20 and XV70-3 in terms of TCA-soluble radioactivity and electrical conductivity were signigicant ($P < 0.01$).

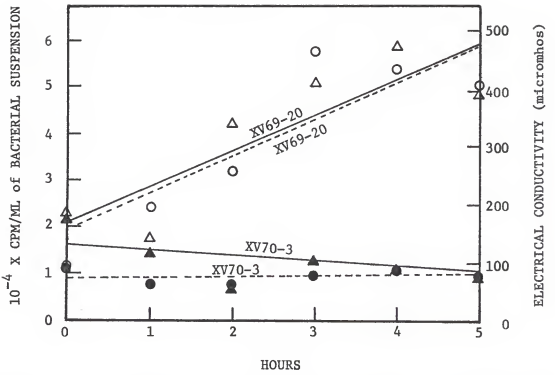

Fig. 15. Trichloracetic acid-soluble radioactivity in suspensions of XV69-20 (△) and XV70-3 (▲) removed from the intercellular spaces of half-leaves of 10-R pepper plants. Conductivity measurements of 10-R half-leaves inoculated with XV69-20 (○) and XV70-3 (●) are indicative of the progress of the hypersensitive and the susceptible responses. Regression line equations for TCA-soluble radioactivity are y=21,354 + 7,612x for XV69-20 and y=16,951 - 1,817x for XV70-3. Regression line equations for TCA-soluble radioactivity are represented by solid lines, and regression line equations for conductivity measurements are represented by broken lines. Based on regression analysis, overall differences between XV69-20 and XV70-3 in terms of TCA-soluble radioactivity and electrical conductivity were significant (P=0.001).

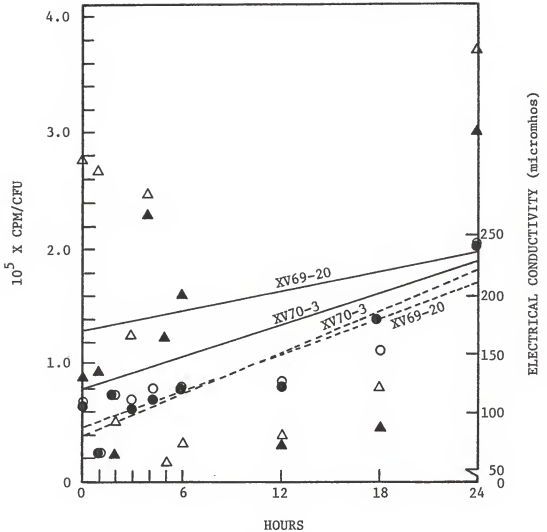

Fig. 16. Rates of protein synthesis in vitro by XV69-20 (△) and XV70-3 (▲) after removal from leaves of ECW pepper plants. Conductivity measurements of ECW half-leaves inoculated with XV69-20 (○) and XV70-3 (●) are indicative of the progress of the susceptible response. Regression line equations for the rates of protein synthesis are y=0.0000131 + 0.00000027x for XV69-20 and y=0.000008 + 0.00000045x for XV70-3. The regression line equations for rates of protein synthesis are represented by solid lines, and regression line equations for conductivity measurements are represented by broken lines. Based on regression analysis, overall differences between XV69-20 and XV70-3 in terms of electrical conductivity and protein synthesis were not significant ($P > 0.05$).

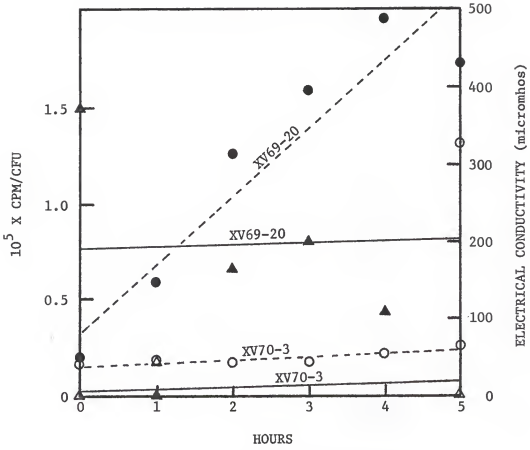

Fig. 17. Rates of protein synthesis in vitro by XV69-20 (▲) and XV70-3 (△) after removal from leaves of 10-R pepper plants. Conductivity measurements of 10-R half-leaves inoculated with XV69-20 (●) and XV70-3 (○) are indicative of the progress of the hypersensitive and the susceptible responses. Regression line equations for rates of protein synthesis are y=0.0000077 + 0.00000078x for XV69-20 and y=0.0000002 + 0.00000011x for XV70-3. Regression line equations for rates of protein synthesis are represented by solid lines, and regression line equations for conductivity measurements are represented by broken lines. Based on regression analysis, overall differences between XV69-20 and XV70-3 in terms of electrical conductivity were significant (P=0.0001). Overall differences in terms of protein synthesis could not be analyzed due to insufficient data. However, the regression of protein synthesis on hours was not significant ($P > 0.05$) for each isolate.

the plant during the interaction. Experiments were designed to remove possible extracellular radioactive protein in the 0.04-ml bacterial suspension from the intercellular spaces of pepper leaves.

Half-leaves of 10-R plants were inoculated with both isolates and labeled amino acids, as described for net protein synthesis experiments. Bacteria were removed from each leaf according to method A, the leaf was discarded from the test tube, and the suspension was centrifuged again at 8700 g for 4 min. When the centrifuge stopped, the supernatant was gently poured through a GA-6 membrane filter, which was then rinsed with 5.0 ml of deionized water. Three ml of deionized water were added to each tube, and the pellet was resuspended in the water by agitation on a Vortex mixer, and tubes were placed back in the centrifuge at 8700 g for 4 min. The supernatant was poured through the same GA-6 membrane filter, which was rinsed well with 10 ml of deionized water. The filter paper was rolled and inserted into the original tube so that the filter surface in contact with the bacteria was enclosed in the center of the roll. Then, 0.5 ml of deionized water was added to the test tube and the tube was placed on a Vortex mixer at high speed for 10 sec. Then, 0.04 ml of the resulting suspension was dropped onto a premoistened GN-6 membrane filter. The filter was rinsed with 5 ml of deionized water, and put into a cocktail vial containing 10 ml of Ready-Solv^{R} HP cocktail. After standing overnight, each vial was agitated on a Vortex mixer for 5 sec, and then placed in a liquid scintillation counter. Another 0.04 ml of each 0.5-ml suspension was extracted with TCA to determine net intracellular protein synthesis.

Comparison of two elements, protein synthesis and radioactive pools (presumably mostly amino acids), between XV69-20 and XV70-3 in 10-R leaves

revealed similar data. These elements increased slightly over a 4.5-hour period for each isolate. However, electrolyte leakage increased substantially only in leaves containing XV69-20 (Fig. 18).

Photometric Assay for Antimicrobial Agent Sensitivity

The sensitivity of X. vesicatoria to antimicrobial agents was determined by the photometric assay, which was based on procedures outlined by Kavanagh (30). Stock solutions of chloramphenicol and streptomycin sulfate were prepared at a concentration dependent on the desired high endpoint in a two-fold dilution series, and stock solutions were sterilized by passage through a 0.45 micron membrane filter. Then, 0.5 ml of each dilution was transferred to each of two sterile 125-ml sidearm flasks. A bacterial suspension was added to 200 ml of sterile nutrient broth to obtain an OD of 0.3 at a wavelength of 600 nm in a Spectronic 20. A constant-volume pipetting device was used to dispense 9.5 ml of the suspension aseptically into each 125-ml sidearm flask. An initial measurement of OD was completed on all flasks. Flasks were put into a reciprocating shaker (120 cycles/min) at room temperature. Additional measurements of OD as well as viable cell counts were taken at specific time intervals over a 24-hour incubation period. The values for the duplicates were averaged, and are presented in Fig. 19, 20, and 21.

Chloramphenicol and streptomycin at a concentration of 512 mcg/ml, or more, produced little or no increase in turbidity indicated by OD measurements during the first 8 hours. This similarity was evident when the effects of growth were expressed as a percentage of growth of the control (Fig. 21). Regression lines, calculated from data expressed in this manner, were similar for both antimicrobial agents, indicating similar kinetics of growth inhibition.

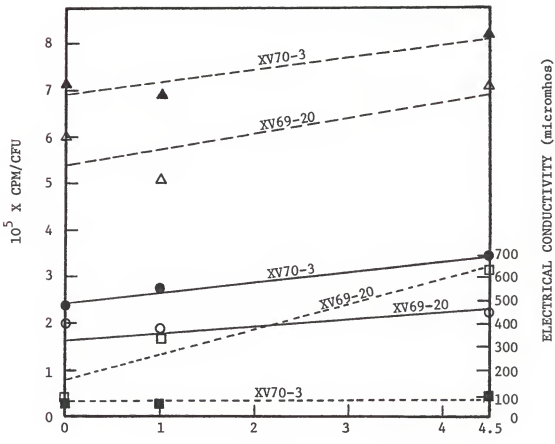

Fig. 18. Intracellular protein synthesis in XV69-20 (○) and XV70-3 (●) and radioactive pools in XV69-20 (△) and XV70-3 (▲) in 10-R pepper leaves. Conductivity measurements of 10-R half-leaves inoculated with XV69-20 (□) and XV70-3 (■) are indicative of the progress of the hypersensitive and the susceptible responses. Regression line equations for intracellular protein synthesis are y=0.000019 + 0.0000007x for XV69-20 and y=0.000024 + 0.0000023x for XV70-3. Regression line equations for radioactive pools are y=0.000054 + 0.0000034x for XV69-20 and y=0.000069 + 0.000069 + 0.0000027x for XV70-3. Regression line equations for intracellular protein synthesis are represented by solid lines, equations for radioactive pools are represented by long-segment broken lines, and the equations for conductivity measurements are represented by short-segment broken lines. Based on regression analysis, overall differences between XV69-20 and XV70-3 in terms of intracellular protein synthesis and radioactive pools were not significant ($P > 0.05$). However, overall differences in electrical conductivity between each isolate were significant ($P=0.0001$).

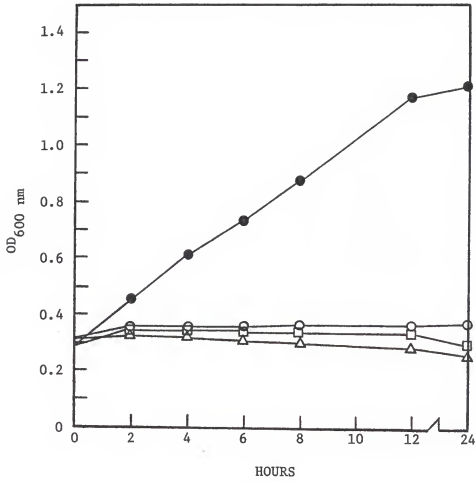

Fig. 19. Growth of XV69-20 in nutrient broth cultures containing chloramphenicol at concentrations of 0 mcg/ml (●), 16 mcg/ml (○), 64 mcg/ml (□), and 512 mcg/ml (△). Chloramphenicol was added at zero time.

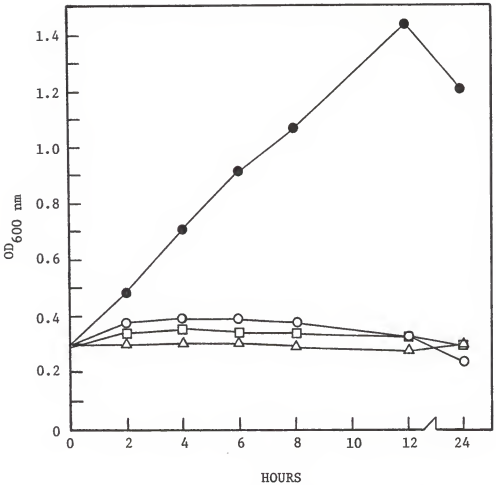

Fig. 20. Growth of XV69-20 in nutrient broth cultures containing streptomycin sulfate at concentrations of 0 mcg/ml (●), 16 mcg/ml (○), 64 mcg/ml (□), and 512 mcg/ml (△). Streptomycin sulfate was added at zero time.

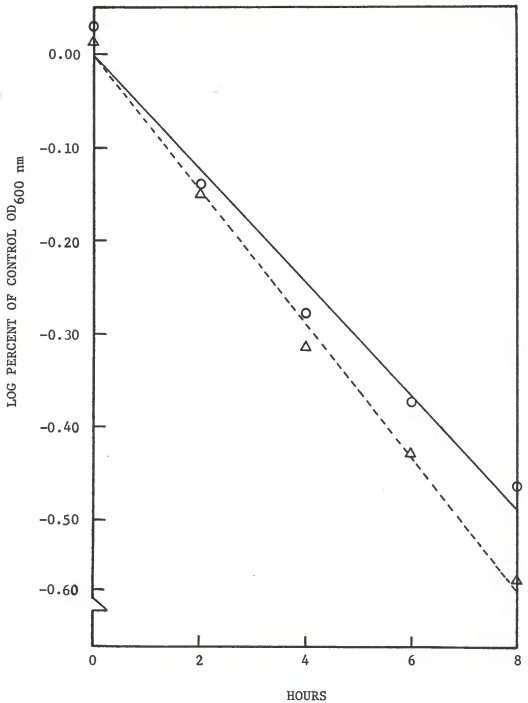

Fig. 21. Rates at which 512 mcg/ml solutions of chloramphenicol (O) and 512 mcg/ml solutions of streptomycin sulfate (Δ) inhibit growth of XV69-20 in vitro. Antimicrobial agents were added at zero time. The regression line equations for inhibition of growth are y=-0.0008 - 0.061x in the presence of chloramphenicol (solid line) and y=-0.009 - 0.072x in the presence of streptomycin sulfate (broken line).

The Effects of Antimicrobial Agents on Protein Synthesis In Vitro

Chloramphenicol or streptomycin sulfate, two antimicrobial agents which inhibit protein synthesis by alteration of ribosome function (43), were added to cultures of X. vesicatoria, and the rates of protein synthesis in the presence of the antimicrobial agents were determined. Nutrient broth cultures of XV69-20 were prepared and adjusted to a concentration of 5×10^8 cells/ml. The cultures were incubated at 30 C in an oscillating waterbath for 30 min. Then, a sample was removed from each flask (zero time) for pulse-labeling, and the OD of each culture was determined. Then, solutions of chloramphenicol and streptomycin sulfate were added to the appropriate flasks to yield 500 mcg/ml of each antimicrobial agent in solution. Additional samples were taken and the optical densities were determined at specific time intervals. Curves for growth and protein synthesis were developed (Fig. 22).

Turbidity in control flasks increased slightly during the test period, but turbidity in the flasks receiving chloramphenicol and streptomycin did not increase. Chloramphenicol and streptomycin sulfate completely inhibited protein synthesis within 10 min after their addition to the cultures.

The Effects of Chloramphenicol and Streptomycin sulfate on Protein Synthesis in XV69-20 In Vivo

In a previous report (39) chloramphenicol inhibited the hypersensitive response more rapidly than streptomycin sulfate in pepper leaves inoculated with X. vesicatoria. It was hypothesized that this differential effect may be due to a difference in the rate of inhibition of protein synthesis in XV69-20 in vivo by the two antimicrobial agents. An experiment was designed to test this hypothesis.

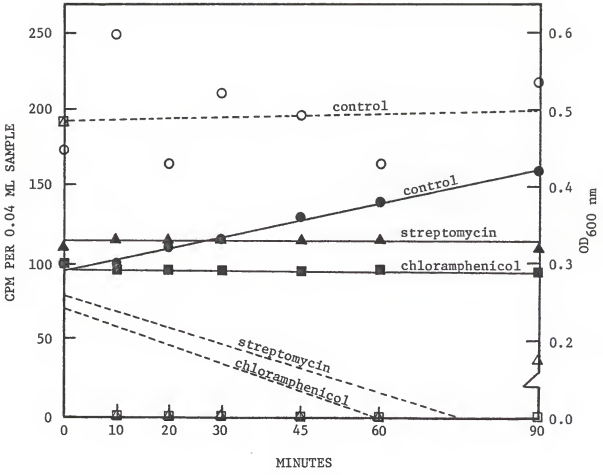

Fig. 22. Protein synthesis (open symbols) and turbidity measurements (shaded symbols) of XV69-20 in nutrient broth cultures containing chloramphenicol at 500 mcg/ml (□,■), containing streptomycin sulfate at 500 mcg/ml (○,●), or containing no antimicrobial agent (△,▲). The antimicrobial agents were added at zero time. Regression line equations for protein synthesis are represented by broken lines, and regression line equations for turbidity measurements are represented by solid lines.

Inocula were suspended in nutrient broth that contained ^{14}C-amino acids and the antimicrobial agent under study. Using a half-leaf experimental design, each half-leaf was inoculated with a bacterial suspension. The control side of the half-leaf was uppermost on the plant to protect the control side from contamination with excess inoculum containing any antimicrobial agent from the experimental side of the half-leaf. The last two inoculated leaves were excised at zero time from test plants, and the plants were returned to the Sherer growth chamber. Then, additional leaf samples were removed from the test plants at 0.5, 1.0, 1.5, and 2.0 hours after inoculation. Bacteria were removed from the inoculated leaves by method B, and net protein synthesis was determined.

The effects of chloramphenicol and streptomycin sulfate on net protein synthesis in XV69-20 *in vivo* are presented in Fig. 23 and 24. Bacteria treated with chloramphenicol exhibited a much greater reduction in bacterial protein synthesis *in vivo* than leaves treated with streptomycin. The relative ineffectiveness of streptomycin on the inhibition of net protein synthesis was associated with a marked increase in electrical conductivity in both treated and control half-leaves (Fig. 24). The substantial effect of chloramphenicol on the inhibition of net protein synthesis was associated with a substantial difference in electrolyte leakage between the control and the treated half-leaves past 2 hours after inoculation.

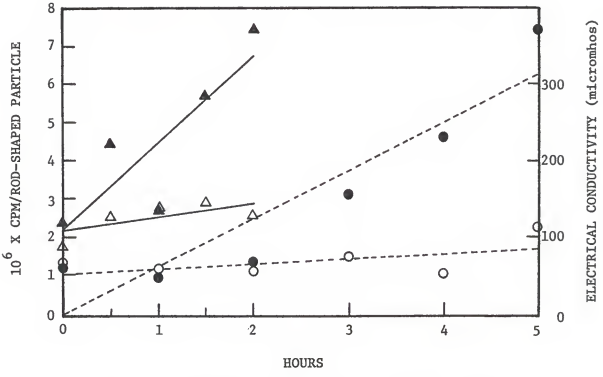

Fig. 23. Protein synthesis by XV69-20 in 10-R pepper leaves in the presence (△) or absence (▲) of 500 mcg/ml solution of chloramphenicol. Conductivity measurements of 10-R half-leaves inoculated with XV69-20 and chloramphenicol (○) or inoculated with XV69-20 alone (●) are indicative of the progress of the hypersensitive response. Regression line equations for protein synthesis are represented by solid lines, and regression line equations for conductivity measurements are represented by broken lines.

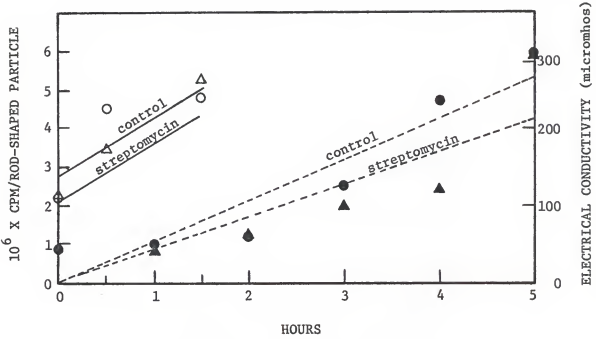

Fig. 24. Protein synthesis by XV69-20 in 10-R pepper leaves in the presence (△) or absence (○) of 500 mcg/ml solution of streptomycin sulfate. Conductivity measurements of 10-R half-leaves inoculated with XV69-20 and streptomycin sulfate (▲) or inoculated with XV69-20 alone (●) are indicative of the progress of the hypersensitive response. The regression line equations for protein synthesis are represented by solid lines, and regression line equations for conductivity measurements are represented by broken lines.

DISCUSSION

In many reports concerning protein synthesis in bacteria, bacterial cell numbers are assumed to be constant, and data are expressed as counts per minute (cpm)/ml of bacteria. When cell number is inconstant, data are often expressed as cpm/OD unit or cpm/mg of protein. There was little sample available in this work for protein determination using the Lowry method (36), since sample size was usually less than 0.5 ml. Photometric determination of the number of bacterial cells with a micro-cell adapter for a Spectronic 20 was not feasible either because much host cellular debris occurred in some samples. Some bacterial suspensions were also dark-colored which would have altered photometric analysis. The variation in bacterial cell numbers between samples in this work dictated that radioactive counts in some experiments had to be expressed in cpm/colony-forming unit (cpm/cfu) or cpm/rod-shaped particle. Although variation in determinations of cfu was inherent (3), it was not thought that it would influence results.

Determination of blank counts was a major problem due to the inconsistency of bacterial cell numbers among samples. Theoretically, a blank should be prepared for each sample in order to determine extraneous counts; but in addition to being expensive because a typical time course experiment consisted of nearly 30 samples, there was not always enough sample left to prepare a blank. In the experiments in which isotope was injected into pepper leaves with the bacteria, the data were expressed as total counts per min per cfu (blank counts were not subtracted from total

counts, but counts indicating background radiation were subtracted). Data expressed in this manner followed a trend similar to data expressed as net counts per min per cfu (blank counts were subtracted from total counts for net counts). Most experiments on net protein synthesis did not involve preparing blanks at all sampling times. At random time intervals, blanks were prepared to check the net cpm vs. the total cpm relationship.

Whole-cell bacterial studies using radioactive isotopes seem to be characterized by low count rates for the labeled samples, so most samples were counted to 2.0% error. A difference in count rate between sample and blank of more than 2.0% was considered to be a detectable difference.

Usually, isotopes are taken up by plant roots or through the petiole of excised leaves in radioisotope studies. In this research the radioisotope was injected into the intercellular spaces of the pepper leaves simultaneously with the bacterial parasite. Although it is a risky technique for the investigator, it seems to have several advantages. One, the label is placed immediately where it needs to be (in the intercellular space). Two, the label probably does not become chemically altered as amino acids are regularly found in the intercellular fluid of pepper leaves (53). Third, the host has less time to incorporate the label into its proteins.

Protein synthesis by the two isolates of X. vesicatoria in pepper leaves was determined using two methods. Each method has inherent problems. The first method involved injection of labeled amino acids into pepper leaves simultaneously with the bacteria. Considering the minimal amount of radioactivity which was recovered from the intercellular spaces

when compared to the activity of the isotope which was injected initially, it was assumed that the pepper cells must have taken up much of the ^{14}C-amino acid mixture. Hence, the quantity of isotope which was available to each bacterium as they induced pathogenic reactions was not clear. Also, bacterial protein, which was analyzed using this method, may have included labeled proteins of host origin.

In the second method (pulse-label method), the ^{14}C was not added to bacteria until the bacteria had been removed from leaf tissue. Alterations in growth or injuries sustained by the bacteria during extraction may have altered subsequent protein synthesis. The pulse-label method eliminated several technical difficulties encountered in the first method, but the data obtained was quite variable. The erratic nature of this data may have been attributable to an induced lag phase precipitated during extraction of bacteria from leaf tissue. Induced lag phases have been reported (29). This extraction process included dilution of intercellular bacteria in distilled water followed by centrifugation and resuspension. However, the similar data from experiments using either method indicate that these considerations are probably not significant.

Despite the unique pathogenic reactions induced by either isolate in different host-parasite combinations, no differences in protein synthesis detected with either method were documented. Protein synthesis increased over a 24-hr period for each isolate in the ECW host. This increase was associated with an increase in electrolyte leakage. In the incompatible interaction of XV69-20 and 10-R, no increase in protein synthesis was detected in XV69-20, although there was a substantial increase in electrolyte leakage. There may have been a release of bacteriostatic agents during the period of electrolyte leakage in this incompatible reaction (57).

Numerous studies have been conducted on protein synthesis in host-fungal interactions. In many of these studies the origin of synthesized protein was not determined. A unique system to differentiate the components of an interaction was developed by Martin and Ellingboe (38). They used the pathogen Erysiphe graminis f.s.p. tritici, the causal agent of powdery mildew in wheat. The ectoparasitic portion was embedded in parlodion, and removal of the parlodion from the host removed the entire ectoparasitic portion of the fungus from the plant surface, leaving the haustoria (a small percentage of the mass of the fungus) behind in the plant. However, these two scientists did not evaluate protein synthesis of the parasite in their system.

The origin of synthesized protein, bacterium or host, was of concern to Hildebrand and Schroth (26). They removed bacteria from leaves to measure the beta-glucosidase synthesized in vivo. The enzyme that they measured was considered to be present in the host-parasite relationship. The enzyme within the bacterial cell was measured as well as that excreted by the bacterial cell and/or the host cell. Beta-glucosidase was bound loosely to bacterial cells and could have been lost during the extraction of bacteria from leaves. They used related organisms which did not synthesize beta-glucosidase to determine the contribution of the host.

The origin of the protein synthesized was not determined in this research work. However, two pieces of evidence supported the presumption that most of the extracted protein was synthesized by the bacterium. First, the antimicrobial agents altered the net protein synthesized. Second, the pulse-label technique precluded protein synthesis by the host cell.

Qualitative analysis of the protein(s) synthesized might be beneficial in separating bacterial protein from plant protein. Certain bacterial proteins are known to be low in cysteine (48). This could be used as evidence of protein origin, but such evidence would not be conclusive.

A comparison of two elements, "intracellular protein" and total radioactivity ("intracellular protein" plus "amino acid pools"), in the interactions between each isolate and 10-R was not distinctive. An increase in the capacity for protein synthesis corresponded with a similar increase in total intracellular radioactivity for each isolate in 10-R. The size of amino acid pools seems to be inversely proportional to to the rate of biosynthesis (15). An expanded amino acid pool would indicate a declining rate of protein synthesis, and vice versa. No firm conclusions about pool sizes can be drawn from this data because an analysis of amino acid uptake was not implemented in the routine manner.

In uptake experiments the cells under study are diluted in a medium free of the radioactive precursor, filtered rapidly (19, 61), and processed at the time of sampling, so that the measurement of intracellular radioactivity reflects that which was present at the sampling time. In these uptake experiments bacteria remained exposed to the radioactive precursor in an aqueous medium for approx 30 min after the sampling was initiated. Hence, some amino acids and proteins may have been lost during the extraction of bacteria from the leaves and subsequent washing, since leakage of amino acids across a bacterial cell membrane has been described (1).

The uptake experiment was originally designed to demonstrate that the increase in protein synthesis observed in the interaction of XV69-20 and 10-R was bacterial in origin. The suspension removed from the

intercellular spaces of the leaves was washed to remove any labeled water-soluble proteins and labeled free amino acids present in the intercellular fluid. It was presumed that the observed increase in protein synthesized in the incompatible interaction represented a corresponding increase in intracellular protein because any protein present in the intercellular fluid had already been washed away. The origin of these intracellular proteins could have been host and/or bacterium, but these proteins probably were proteins of bacterial origin. However, they could have originated from the host because these proteins could have adsorbed to the bacterial cell surfaces and remained intact during washing.

Von Broembsen and Hadwiger (62) and Martin and Ellingboe (38) have measured rates of protein synthesis and ^{32}P transfer, respectively, in compatible and incompatible host-parasite interactions. Von Broembsen and Hadwiger observed an increased rate of protein synthesis for certain incompatible combinations from 6 hr until 18 hr after inoculation when the experiment was terminated. The hypersensitive response was not detectable cytologically until 48 hr after inoculation in their Linum usitatissimum-Melampsori lini system. The elevated levels of net protein synthesis were attributed to the interaction of host and parasite, not specifically to either one. Protein synthesis in compatible interactions remained either constant or decreased over the 18-hr period.

Using the Triticum aestivum-Erysiphe graminis system, Martin and Ellingboe's (38) observations were attributed primarily to the parasite. Supposedly, a "collapse" of the parasite occurred 22 hr after inoculation. The increased transfer of ^{32}P from the host to the incompatible parasite began 8 hr after inoculation and peaked at approx 20 hr after inoculation. At 22 hr after inoculation the rate of ^{32}P transfer to the "collapsed"

incompatible parasite decreased to the rate observed in the compatible interactions. The results obtained by this research team using host-fungal systems did not correlate with the data reported for the C. annuum-X. vesicatoria system. This investigator was not able to demonstrate a different rate of protein synthesis for the incompatible vs. the compatible interaction in this system.

Martin and Ellingboe (38), and von Broembsen and Hadwiger (62) did not report a crucial element in their research study—establishment of a similar metabolic rate among the races of any parasite selected for comparative studies. This crucial similarity was demonstrated in the work with C. annuum and X. vesicatoria.

The two isolates XV69-20 and XV70-3 differed only in the gene(s) governing avirulence in specific near-isogenic C. annuum hosts. Based on similar growth rates and similar rates of protein synthesis in vitro, any substantial difference in net protein synthesis should not be attributed to a difference in the overall metabolism of each isolate.

Protein synthesis determinations in isolate XV69-20 were extended to experiments relating the length of the induction period in 10-R to the rate of inhibition of protein synthesis in XV69-20 by streptomycin and chloramphenicol. Earlier research revealed rather constant low levels of protein synthesis in XV69-20 in an incompatible interaction with 10-R.

Two modifications were made in the procedure used for the induction period experiments involving the effect of each antimicrobial agent on protein synthesis in vivo. First, sampling at "zero time" was taken immediately after injection of the bacterial suspensions into appropriate leaves, rather than waiting until the inoculated leaves were no longer visibly water-soaked, which is customary for "zero time." This change

prevented a period of time in which the antimicrobial agent, that was injected simultaneously with the bacteria into leaves, could alter the host-parasite interaction. Second, XV69-20 was suspended in nutrient broth in an attempt to duplicate the conditions of the in vitro experiments, and to increase the incorporation of the ^{14}C label into protein over the level observed in previous experiments. It is possible that protein synthesis was not optimally supported in the deionized water suspensions of X. vesicatoria used in other experiments.

No appreciable increase in turbidity was noted for cultures of X. vesicatoria at 10 min after the addition of an equal concentration (512 mcg/ml) of either chloramphenicol or streptomycin sulfate. The effects of these two antimicrobial agents on culture turbidity were consistent with information involving other bacteria (14). Furthermore, inhibition of protein synthesis within 10 to 15 min after adding a 500 mcg/ml solution of either chloramphenicol or streptomycin to X. vesicatoria cultures was also consistent with results of similar experiments using Escherichia coli (1, 64).

Differentiation of the effects of chloramphenicol and streptomycin on bacteria may be obtained if close analyses of changes in protein synthesis were undertaken during the first 10 min of incubation. Some detectable protein synthesis after the addition of streptomycin to growing cultures of E. coli was documented (1, 29). Moreover, in some experiments involving chloramphenicol and E. coli, an immediate inhibition of protein synthesis occurred (29, 64). A very rapid inhibition of protein synthesis by chloramphenicol in cells of Staphylococcus aureus has been reported (20). In experiments in which chloramphenicol and streptomycin were added simultaneously to cultures of E. coli, apparently

chloramphenicol interacted more rapidly with cells of E. coli than streptomycin sulfate to inhibit rates of protein synthesis and population growth (29). Hurwitz et al. (28) reported that chloramphenicol prevented the synthesis of protein necessary for streptomycin to exert its lethal effect on a cell. However, Stern et al. (59) demonstrated that streptomycin was lethal to E. coli without the occurrence of protein synthesis.

The results of these experiments with chloramphenicol and streptomycin supplement earlier work (39) on viability and respiration in chloramphenicol-treated and streptomycin-treated cultures of X. vesicatoria. Chloramphenicol was considered to be a bacteriostatic agent (44), whereas streptomycin was considered a bactericide (51). The effects of chloramphenicol can be reversed by washing (44), but the effects of streptomycin are considered irreversible (14). Meadows (39) determined that essentially the whole population of X. vesicatoria was lethally affected within 2 to 3 min after exposure to 500 mcg/ml of streptomycin. This was determined from samples of X. vesicatoria that were dilution-plated at specific intervals of time after treatment.

The characterization of the effects of streptomycin on X. vesicatoria corresponded to observations made by Dubin (15) with E. coli. Dubin et al. (15) observed that viable counts of E. coli treated with streptomycin sulfate began to decrease at the same point that a decrease in protein synthesis occurred. Furthermore, Dubin (15) correlated this decrease in viability with a concomitant decrease in oxygen consumption by E. coli treated with streptomycin. Meadows (39) observed a similar decrease in respiration after streptomyin was added to a growing culture of X. vesicatoria. Therefore, a lethal effect on each bacterium was associated with parallel decreases in protein synthesis and oxygen uptake.

The relative effects of chloramphenicol and streptomycin sulfate on protein synthesis in X. vesicatoria were notably different in vivo than in vitro. Streptomycin was much less effective than chloramphenicol in the inhibition of bacterial protein synthesis in vivo. The relative ineffectiveness of streptomycin in vivo may be influenced by the interaction between this agent and the pepper leaves. Existing evidence lends credence to this possibility. In studies of cation exchange phenomena with streptomycin and metallic cations, Goodman and Goldberg (23) postulated that adsorption by a plant's colloidal system as well as movement of an adsorbed compound within plant tissue is influenced by the type and amount of endogenous cations present in plant tissue. Crowdy and Pramer (10) found a distinct difference between the concentration of chloramphenicol and streptomycin in the expressed sap (tissue fluid forced out under pressure) and in the water extracts (trituration of leaves with distilled water) of plant tissues. The concentrations of chloramphenicol in the sap and in the water extracts were almost equal. The concentration of streptomycin in the sap was twice the concentration in the water extracts. Streptomycin is a cation, whereas chloramphenicol is an electrically neutral molecule.

Pramer (46) noted that a 12-minute exposure of the alga, Nitella clavata, to streptomycin resulted in equal concentrations of the agent within and without the algal cells. Exposure of the alga to streptomycin beyond 12 min revealed that the alga was accumulating it against a concentration gradient. Chloramphenicol was detected within the cell only after exposure of the alga to chloramphenicol for 2 hr. A 24-hr treatment with chloramphenicol produced an intracellular concentration of only one-half of the extracellular concentration. He concluded that absorption of

chloramphenicol appeared to depend on simple diffusion. Crowdy and Pramer (10) compared the concentration of streptomycin and chloramphenicol in the leaf saps of different plants. The concentration of streptomycin in the leaf sap of tomato was ten times greater than the concentration of chloramphenicol. The concentration of both agents in the leaf sap of cabbage was equivalent.

Meadows (39) noted that the concentration of streptomycin in water extracts of pepper leaves that were hypodermically injected with streptomycin was the same at 8 hr after injection as it was immediately after injection. However, the concentration of streptomycin in the leaf sap (cell sap) was not determined. A quantitative bio-assay for streptomycin concentrations in the intercellular fluid, water extract, and cell sap of pepper leaves would be beneficial to determine whether any interaction between streptomycin and the host may influence the observed effect of streptomycin on X. vesicatoria in vivo.

The differences noted between the effects of streptomycin on bacteria in vivo and in vitro may be explained by study of the uptake of streptomycin by E. coli and the interaction of streptomycin with the ribosomes of E. coli. Extrapolations to X. vesicatoria are probably valid because the sensitivities of phytopathogenic bacteria and other bacteria to antimicrobial agents were similar, as Waksman (63) asserted. Three consecutive phases of uptake characterized the interaction of E. coli and streptomycin (27). Electrostatic binding of streptomycin to the bacterium occurred in the first phase. This phase was very rapidly completed in 1 min or less (2). The second phase was characterized by slow uptake of streptomycin. The third phase involved an energy-dependent rapid uptake period, lasting approx 10 to 20 min.

A hypothetical sequence of events described herein may occur upon injection of X. vesicatoria and streptomycin into pepper leaves. Right after entry into the intercellular spaces the mesophyll cells may absorb streptomycin actively. Streptomycin molecules may compete for cation-absorbing sites and become ionically-bound with host constituents. The concentration of streptomycin in the intercellular spaces may decrease with time. Since inhibition kinetics are dependent on agent concentration (29), the rate of inhibition could be decreased due to the reduced agent concentration in the intercellular spaces.

Furthermore, there could be interaction between the antimicrobial agent and the bacterium, too. Since ionic bonding is involved in the first stage of streptomycin uptake in E. coli, conditions could exist in the pepper plant to interfere with streptomycin uptake by X. vesicatoria. The population of X. vesicatoria injected with streptomycin sulfate was approx 6×10^8 cells/ml, more than the concentration necessary for confluent necrosis to occur (less than 1.0×10^8 cells/ml). Therefore, if one-half of the bacterial population had absorbed streptomycin in a lethal amount, a sufficient number of bacteria could remain to induce a confluent HR. However, electrical conductivity patterns were quite similar in streptomycin-treated half-leaves and control half-leaves. If a large portion of the bacterial population in streptomycin-treated half-leaves had been affected, the delay in appearance of the HR would have been greater than that observed. In previous experiments with the C. annuum-X. vesicatoria system, the time of appearance of HR depended on the dose of inoculum used. Therefore, streptomycin probably entered many of the bacterial cells in these experiments.

It is possible that the effects of streptomycin on some of these cells could have been reversed *in vivo*. One example of the reversibility of streptomycin action on bacterial cells involved the interaction of streptomycin and bacterial ribosomes. Streptomycin interacted with both free (initiating) and complexed (preformed) ribosomes (polysomes) in *E. coli* (14). In an *E. coli* cell undergoing protein synthesis, most ribosomes were complexed (14). The effects of streptomycin were much greater on free rather than complexed ribosomes (14). An interaction of streptomycin with complexed ribosomes slowed down polypeptide elongation, but did not stop it (14). Under physiological conditions the interaction of streptomycin with complexed ribosomes was probably reversible (14). Thus, conditions could exist in the pepper host (but not in nutrient broth) which reduce the effect of streptomycin on complexed ribosomes. Other examples of the reversal of streptomycin action exist (24, 45, 47).

The postulate that host effects may account for the ineffectiveness of streptomycin in the inhibition of *X. vesicatoria* in pepper leaves was not supported conclusively by experimental data obtained in this work. Evidence that the ability of an antimicrobial agent to inhibit *X. vesicatoria* and to delay the HR in this system was related to the effects of the antimicrobial agent on bacterial protein synthesis *in vivo*.

Considering existing evidence and the data involving *in vitro* and *in vivo* effects of streptomycin sulfate and chloramphenicol on *X. vesicatoria*, the value in the determination of "induction periods" using streptomycin in certain hosts should be questioned. The outcome of an induction period may be the result of interaction of many variables pertaining to the host, the parasite, and/or their interaction. Therefore, the three-phase concept of the bacterial-induced HR should be reevaluated.

SUMMARY

The first hypothesis, that the relative effectiveness of an antimicrobial agent in the determination of induction periods for the bacterial-induced hypersensitive response is correlated with the rate at which it inhibits protein synthesis in the bacterium *in vivo* but is not correlated necessarily with inhibition of protein synthesis *in vitro*, was supported by the data obtained in this investigation. Both chloramphenicol and streptomycin seemed to be equally effective in the inhibition of protein synthesis of XV69-20 *in vitro*. Chloramphenicol inhibited protein synthesis quite effectively *in vivo*, but streptomycin did not. An influence of the host on the streptomycin was postulated to explain the ineffectiveness of streptomycin on protein synthesis *in vivo*.

The second hypothesis, that cells of *X. vesicatoria* are metabolically stimulated in an incompatible interaction more than in a compatible interaction which is reflected in an increase in total bacterial protein or in an increased rate of protein synthesis by the bacterium, was not supported by the data obtained in this investigation, though.

No increase in protein synthesis was detected in the incompatible vs. the compatible interactions of 10-R and each isolate. Substantial electrolyte leakage was observed in one stage of the incompatible interaction of 10-R and XV69-20 without a corresponding increase in protein synthesis, which may indicate a bacteriostatic substance that was released during the hypersensitive response. In the compatible interactions of each isolate and ECW, an increase in the rate of protein synthesis was detected

with a parallel increase in electrolyte leakage. Study of the interactions of phytopathogenic bacteria and their hosts in this research was a novel approach to the problem of the study of rates of biosynthesis in compatible and incompatible host-parasite interactions.

Further investigation of the bacterial-induced hypersensitive response with appropriate variations of the procedures used in this research to explore the role of protein synthesis in each interaction is recommended. Appropriate variations include the use of nutrient broth or another medium besides deionized water to suspend bacteria for any *in vivo* studies, the use of nutrient medium or a buffer when bacteria are removed from leaves, the use of higher concentrations of radioisotope, the extension of the length of the pulse-label period, a reduction of the inoculum concentration to permit the incompatible interaction to occur more slowly, labeling of the host plant prior to inoculation with bacteria, and detection of probable lag phases induced by experimental procedures.

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BIOGRAPHICAL SKETCH

Michael Edward Meadows was born in Birmingham, Alabama, on July 10, 1950. In 1959 his family settled on a farm near DeFuniak Springs in northwest Florida. He was graduated from Walton High School in DeFuniak Springs in 1968. He earned an Associate of Arts degree from Okaloosa-Walton Junior College, located in the neighboring town of Niceville, in 1970. He received the degree of Bachelor of Science in Agriculture in 1973 and the degree of Master of Science in 1976 from the University of Florida in Gainesville, Florida.

In April 1973 he was employed by the Plant Pathology Department of the University of Florida as a laboratory technician. He worked in the Plant Disease Clinic under the direction of Dr. R. S. Mullin and Dr. T. A. Kucharek, where he developed an avid interest in plant pathology. He returned to graduate school in 1974 to pursue an advanced degree in plant pathology. He has been working toward the degree of Doctor of Philosophy since 1976 when he received the degree of Master of Science.

He met his wife Karen while attending the University of Florida, and was married in Gainesville in December 1973. He and his wife have been blessed with two children: Michael, age 4, and Angela, age 2.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert E. Stall, Chairman
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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